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ORIGINAL PAPER

Variation of phenotype, ploidy level, and organogenic potential of in vitro regenerated polyploids of *Pyrus communis*

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Abstract A wide range of phenotypic variation was observed among neopolyploids obtained from the diploid pear cultivar 'Fertility' by in vitro colchicine treatment. The variant plantlets had alterations in leaf characteristics. Neopolyploids had significantly different ratios of leaf length to leaf width compared to the diploid control. Shoot regeneration from leaf explants and rooting ability from in vitro shoots of neopolyploids was examined. Regeneration frequencies of shoots from leaf explants of seven of the nine neopolyploids were significantly decreased compared to the diploid control. The organogenic potential of neopolyploids was highly genotype-dependent for both shoots and roots. Tetraploid clone 4x − 4 failed to regenerate shoots from leaf explants and the pentaploid clone 5x − 2 failed to root from in vitro shoots. The results suggest that polyploidization caused the decrease in or loss of in vitro organogenic potential. Regenerated shoots derived from neopolyploids showed different phenotypes, depending on the ploidy of the donor plant.

Keywords Phenotypic variation · Ploidy · Shoot regeneration · Organogenesis · Totipotency · Somaclonal variation · Pear

Introduction

Polyploidy is widely acknowledged as a major mechanism of adaptation and speciation in plants (Ramsey and Schemske 2002). Polyploidization often causes large-scale genomic reorganizations and is accompanied by a wide variety of phenotypic alterations in morphology, niche preference and fitness characteristics. Polyploids are excellent genetic material for comparative analysis of gene expression and genomic changes after polyploid formation because the exact progenitors are known (Dhooghe et al. 2011). Polyploid plants are also used in functional genomics research (Cheng and Korban 2011; Yang et al. 2011). Polyploid species can have a higher adaptability and increased tolerance to various environmental conditions. Most of the world's biota (our life support system) is polyploid, and life on earth is predominantly a polyploid plant phenomenon (Bennett 2004). Considering the significance and importance of plant polyploidy in agriculture and genomics research, it is understandable that there was considerable interest in developing induced polyploids when mitotic inhibitors were first discovered in the 1930s (Ranney 2006). With the development of plant tissue culture techniques, colchicine has been widely used to induce somatic cell chromosome doubling. A polyploid that has been produced by artificially inducing chromosome doubling is called a neopolyploid (Comai 2005). Neopolyploid production with colchicine has been achieved successfully in many crops (Gao et al. 1996; Shao et al. 2003; Stanys et al. 2004; Wu and Mooney 2002). There is a large amount of literature on the advantages of polyploidy and the success of polyploids in nature. However, optimum ploidy levels seem to vary between different groups, and there are clearly limits to multiplying copies of the nuclear genome before this becomes a liability (Bennett 2004). Polyploids generally differ markedly from their progenitors in morphological, ecological, physiological and cytological characteristics that can contribute both to exploitation of a new niche and to reproductive isolation (Ramsey and Schemske 2002). Phenotypic variations resulting from ploidy level were reported in several

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species (Li et al. 1992; Pontaroli and Camadro 2005). There have been some reports about the relationship between polyploidization of the cell and the capacity for somatic embryogenesis and organogenesis (Coutos-Thevenot et al. 1990; Colijn-Hooymans et al. 1994; Torrey 1967). The degree of variation in phenotype and organogenic ability from explants of neopolyploids in woody crops have not been reported.

Materials and methods

Plant materials and culture conditions

Shoot cultures of the diploid (2x) pear cultivar 'Fertility' (*Pyrus communis* L.) and ten neopolyploids obtained in our laboratory (Sun et al. 2009a) were proliferated and maintained on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) with 1 mg l^{-1} 6-benzylaminopurine (BA) and 0.2 mg l^{-1} indole-3-butyric acid (IBA), 3% sucrose and 0.6% agar, and with the pH adjusted to 5.8 before autoclaving (121°C, 20 min). In vitro shoots, 1.5 cm or longer, were excised from the pear clones of different ploidy and were placed on rooting medium (RM) consisting of 1/2 QL (Quoirin and Lepoivre 1977) medium with 0.5 mg l^{-1} IBA, 1 g l^{-1} polyvinyl alcohol (PVA), 2% sucrose, solidified with 0.6% agar and with the pH adjusted to 5.8 before autoclaving (121°C, 20 min), as previously reported (Sun et al. 2009b).

Phenotype variation of clones of different ploidy

After 30 days on RM, shoots were excised from in vitro rooted plantlets and transferred to the same, but fresh RM medium for another 30 days to be induced to root. For clone 5x − 2, which failed to root after the first 30 days on RM, shoots were also excised and transferred to fresh RM. Changes to the leaf morphology of each clone were investigated. Growth of adventitious roots of each clone (pentaploid 5x − 2 failed to root) was also examined. Petiole length and width at the central part of the petiole, leaf lamina length, width and thickness at the central part of the leaf lamina of polyploids and their diploid control plantlets were measured using a vernier caliper. Two fully developed and matured leaves were randomly collected from 25 plantlets of each ploidy clone for a total of 50 leaves per clone. A leaf index (Tsukaya 2002) was calculated as the ratio of leaf lamina length to leaf lamina width.

Shoot regeneration from leaf explants of neopolyploids and ploidy level analysis of regenerants

Adventitious shoot regeneration from in vitro leaf explants of the diploid control and neopolyploids was examined. Expanding young leaves were excised from actively growing shoots which had been subcultured 4 weeks on MS medium. The excised leaves were wounded by several transverse cuts across the mid-rib, and placed horizontally with the abaxial side up onto various adventitious shoot induction media (SIM). Three experiments were performed to evaluate the effects of ploidy level, SIM, and carbon source and concentration, respectively, on adventitious shoot regeneration.

Experiment 1

Young leaf explants were collected from shoot tips of the original diploid and nine neopolyploid clones and placed on SIM consisting of Nitsch and Nitsch (NN69) medium (Nitsch and Nitsch 1969) with 5 mg l^{-1} BA and 0.5 mg l^{-1} IBA, 30 g l^{-1} sucrose, and 6 g l^{-1}agar (Sun and Sun 1999). For each clone, at least three petri dishes (90 × 15 mm) were prepared, and each petri dish contained ten leaves. All explants were incubated in the dark for 3 weeks before exposure to light. At the end of 7 weeks, the shoot regeneration rate (number of leaf explants regenerating shoots/number of leaf explants inoculated) was determined.

Experiment 2

In order to choose an appropriate SIM for the seven neopolyploids that exhibited poor regeneration in Experiment 1, three shoot induction media were tested: NN69 with 0.5 mg l^{-1} IBA and either 1 or 2 mg l^{-1} thidiazuron (TDZ), and 1/2 MS (half-strength MS macronutrients) with 5 mg l^{-1} BA and 0.5 mg l^{-1} IBA (Table 3).

Experiment 3

The effects of carbon source (maltose or sucrose) and concentration (20, 25, or 30 g l^{-1}) on shoot regeneration of the seven poorly regenerating neopolyploid clones and the diploid control were investigated (Table 4). The other components of the SIM medium were the same as used in Experiment 1.

Replication and leaf explant culture conditions of Experiments 2 and 3 were same as Experiment 1. All experiments were performed 3 times. The ploidy level of regenerants was analyzed using flow cytometry as previously reported (Sun et al. 2009a).

Statistical analysis

Data for all experiments were analyzed to determine whether the residuals met the assumptions underlying the analysis of variance. Homogeneity of variances was tested by an examination of residual plots. Normality was tested

using the Shaprio-Wilk test in SAS PROC UNIVARIATE (SAS Institute Inc. 1990) and examination of box and normal probability plots. Correlation of means and residuals was tested by SAS PROC CORR option SPEARMAN. Type III sums of squares were computed. All analyses of variance were performed using SAS PROC MIXED (Littell et al. 1996). Least-square means separation for this procedure was performed using the Tukey–Kramer honestly significant difference (HSD) method. Mean separation letters for main effects were assigned by the SAS macro PDMIX 800 (Saxton 1998). For all traits, clones were considered a main fixed effect, regardless of the ploidy level.

The morphological leaf and petiole traits were analyzed with clone as the fixed main effect. Shoot within clone and leaf within shoot within clone were the random nested samples and subsamples. For all regeneration experiments, treatment by replicate means were used as the basis of all analyses. The experiments had randomized complete block designs with the replicate experiments designated as the random block effects. In Experiment 1, clone was treated as a fixed main effect. In a second analysis, ploidy was a main effect and clone was treated as a nested factor within ploidy. For Experiment 2 on the effect of different SIM media, the design was a factorial with clone and media as fixed effects. The SLICE option was used to test for the significance of shoot induction media within each neopolyploid clone. For Experiment 3 on the effect of carbon source and concentration, clone and carbon source were analyzed as qualitative fixed effects and concentration was treated as a quantitative factor. The significance of differences among the six carbon source by concentration treatments within each clone were also tested using the SLICE option, analyzing each carbon source by concentration combination as separate qualitative treatments. Least-square main effect means for each clone, carbon source and concentration were also tested.

Results

Tests for normality, equality of variances, and non-correlation of means and variances did not detect serious deviations from the assumptions underlying parametric analysis of variance. The deviations were not correctable by standard transformations. Therefore, original data was used for all analyses.

Phenotype variation of neopolyploids

In this paper, a wide range of phenotypic variation in the neopolyploids was observed. Neopolyploids were divided into two major types according to leaf colour. In type A, the intensity of leaf colour was similar to the diploid control (Fig. 1a), but leaf lamina shape was different, either more broad (Fig. 1b–d) or longer (Fig. 1e) than the diploid, and the petiole was shorter (Fig. 1b–d). In type B, leaf colour was darker (Fig. 1f–i) than that of type A polyploids and the diploid control. Plants of type B neopolyploids were very easy to distinguish visually from type A neopolyploids and the diploid control according to leaf traits (leaf colour and leaf shape). Flow cytometry analysis confirmed that type A neopolyploids were two triploids (3x − 1, 3x − 2) and three tetraploids (4x − 1, 4x − 2, 4x − 3) and type B were one triploid (3x − 3), one tetraploid (4x − 4), two pentaploids (5x − 1, 5x − 2) and one mixploid (4x/6x).

Compared to the diploid control, polyploids had significantly smaller leaf lamina length and petiole length and significantly larger leaf lamina thickness (Table 1). The significance of differences in leaf lamina width and petiole width between diploid and polyploids depended on the clone. The tetraploid clone 4x − 1 possessed the widest leaf lamina, while the triploid 3x − 3 had the narrowest lamina. There were significant differences in the leaf index between polyploids (exclusive of 5x − 2) and the diploid, but polyploid clones 3x − 2 and 3x − 3 had significantly larger values for the leaf index than the diploid, and other polyploids (exclusive of 5x − 2) had significantly smaller leaf index values than the diploid. The leaf morphology results revealed differences in leaf traits of polyploids and the diploid clone. Triploids 3x − 2 and 3x − 3 (regenerants from Mix − 1) had larger leaf index values than the diploid as a result of shorter and narrower leaves than those of the diploid. Other polyploids had smaller leaf index values than the diploid. Therefore, leaf index values significantly greater than or less than the diploid may be used as a preliminary indicator for selection of neopolyploid variants regenerated from the original diploid. Leaf thickness could also be used as a supplementary indicator of polyploidy, because polyploids had significantly thicker leaves than the diploid. The leaf margins of different polyploid clones were also different. Clone 4x − 4 had smooth margins (Fig. 1f), while others were dentated. With the exception of clone pentaploid 5x − 2 (Fig. 1h), the size of the leaf dentation was more pronounced.

Root organogenesis

Shoots (rooted and non-rooted) of plantlets of triploid 3x − 2 (Fig. 2a), 3x − 3 (Fig. 2b), tetraploid 4x − 4 (Fig. 2c), pentaploids (Fig. 2d, e) and mixploid Mix − 1(Fig. 2f) grew well on RM, showing vigorous apical growth, but those of the diploid (Fig. 2g), triploid 3x − 1 (Fig. 2h) and the tetraploids 4x − 1 and, 4x − 2 (Fig. 2i, j) did not grow well on RM, showing cessation of apical

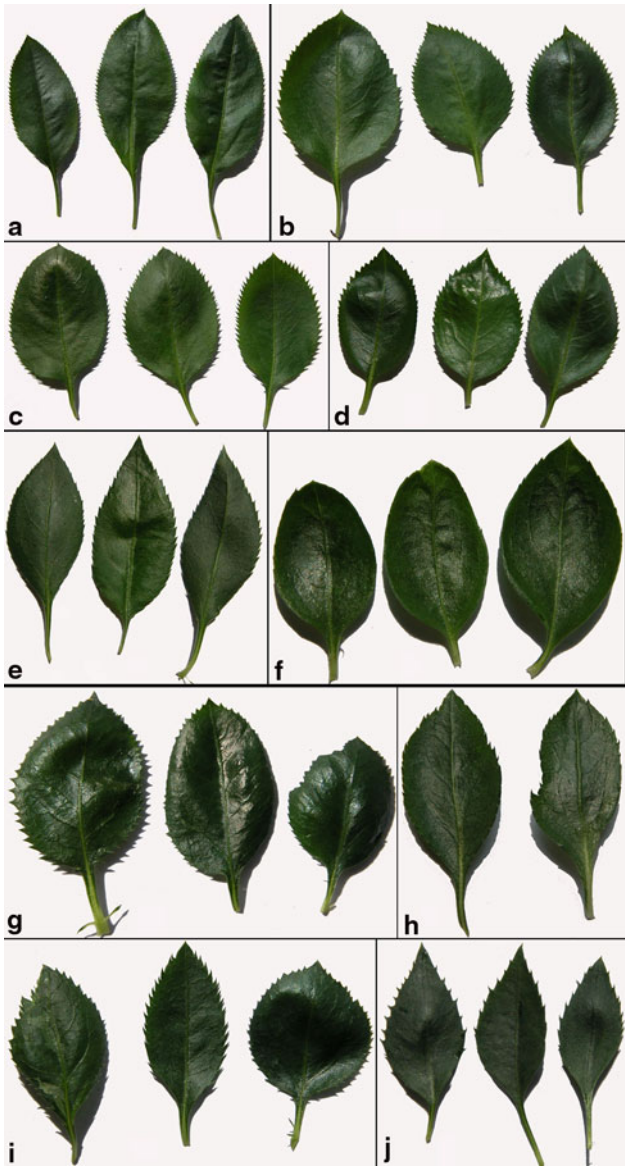

Fig. 1 Leaf morphology variation of different ploidy clones of pear plantlets. **a** diploid cultivar 'Fertility' (control) **b** and **c** tetraploid clones 4x − 1 and 4x − 2 **d** and **e** triploid clones 3x − 1 and 3x − 2 **f** tetraploid clone 4x − 4 **g** and **h** pentaploid clones 5x − 1 and 5x − 2 **i** mixploid clone Mix − 1 **j** triploid clone (regenerated from Mix − 1) 3x − 3

growth. Sun et al. (2009b) reported that the efficiency of in vitro rooting of triploid, tetraploid and mixploid and its diploid control was highly genotype − dependent, with rooting rates of neopolyploids lower than that of the diploid. In this paper, root organogenesis of the polyploidy clones was further examined. Rooting frequencies among different ploidy groups and among genotypes of the same ploidy were significantly different (data not presented), similar to previously reported results (Sun et al. 2009b). Clone 5x − 2 was very difficult to root and failed to regenerate (Fig. 2e) on many different media over a period of 3 years (data not presented). Root growth of different clones on RM was different. The roots of clone 4x − 4 appeared shorter (Fig. 2c) than those of the other polyploids and the diploid, and were very difficult to elongate even over long culture periods and with different rooting media (data not presented). The roots of clone 3x − 1 could grow very long (Fig. 2h), similar to the diploid, but appeared thicker than the diploid. The roots of other polyploids appeared shorter but thicker (Fig. 2a–d, f, i, j) than those of the diploid.

Shoot organogenesis from leaf explants of neopolyploids

Shoot regeneration of the various clones were different on the same SIM (Table 2). Pooling clones of the same ploidy, the diploid (83.3%) regenerated significantly more than the tetraploids (39.3%), and these were significantly more responsive than the pentaploids (13.9%), triploids (11.7%), and the mixploid (6.7%). Regeneration rates of tetraploids 4x − 1 and 4x − 3 were not significantly different from the diploid. Among the tetraploid clones, 4x − 1 and 4x − 3 regenerated equally well, while regeneration of 4x − 2 was significantly lower. Clone 4x − 4 failed to regenerate. The two triploids were not significantly different in regeneration, and the two pentaploids were also not significantly different from each other. These results revealed that polyploidy caused by genome duplication can result in a reduction of in vitro shoot organogenesis, although that may not be true of all tetraploid neopolyploids.

In order to improve the shoot regeneration rates of neopolyploids which had previously not regenerated well (Table 2), the effects of shoot induction medium basal salt composition and carbon source were examined. Differences among clones were significant ($P = 0.0001$), differences among media were not significant ($P = 0.96$), and clone by medium interactions were not significant ($P = 0.52$). When 1 mg l^{-1} or 2 mg l^{-1} TDZ was substituted for 5 mg l^{-1} BA as the cytokinin with NN69 as the basal salt medium (Table 2), the regeneration rate was improved only for 3x − 1 and 4x − 2 (Table 3). When NN69 was used with the higher rate of TDZ, the regeneration rate was significantly reduced for 5x − 1, showing the organogenic process of 5x − 1 was inhibited by excess TDZ. In contrast, the same medium increased regeneration of 3x − 1. When MS medium with half-strength macronutrients was substituted for NN69 basal medium (Table 1), regeneration rates for all tested neopolyploid clones were not improved, with the possible exception of 5x − 1. QL or half-strength QL basal media and a higher concentration of TDZ (4 mg l^{-1}) also had no effect on shoot regeneration ability of the tested neopolyploids (data not presented). Clones 4x − 4 and 3x − 2 failed to regenerate.

Table 1 Comparison of leaf shape among different ploidy clones in pear

Ploidy clone	Leaf lamina length (cm)	Leaf lamina width (cm)	Leaf lamina length/leaf lamina width	Leaf lamina thickness (mm)	Petiole length (mm)	Petiole width (mm)
Mix − 1	16.50 ± 0.51 fg	10.81 ± 0.33 d	1.53 ± 0.13 d	0.14 ± 0.004 b	4.98 ± 0.16 c	0.61 ± 0.02 cd
5x − 1	18.11 ± 0.35 de	11.56 ± 0.29 bc	1.59 ± 0.16 d	0.15 ± 0.001 a	5.19 ± 0.16 bc	0.71 ± 0.01 a
5x − 2	17.45 ± 0.48 ef	9.16 ± 0.33 f	1.94 ± 0.20 b	0.12 ± 0.001 f	6.41 ± 0.27 de	0.55 ± 0.01 ef
4x − 1	19.71 ± 0.50 bc	13.05 ± 0.37 a	1.52 ± 0.11 d	0.13 ± 0.003 c	6.99 ± 0.30 b	0.63 ± 0.01 bc
4x − 2	18.67 ± 0.38 cd	11.62 ± 0.27 bc	1.62 ± 0.19 cd	0.10 ± 0.003 g	5.14 ± 0.13 e	0.59 ± 0.01 de
4x − 3	18.98 ± 0.46 cd	11.06 ± 0.37 bcd	1.74 ± 0.22 c	0.12 ± 0.001 ef	7.06 ± 0.23 b	0.56 ± 0.01 e
4x − 4	18.94 ± 0.50 cd	10.98 ± 0.26 cd	1.74 ± 0.21 c	0.12 ± 0.003 de	5.91 ± 0.18 cd	0.65 ± 0.01 b
3x − 1	17.93 ± 0.46 de	11.32 ± 0.31 bcd	1.59 ± 0.12 d	0.12 ± 0.003 f	5.04 ± 0.22 e	0.56 ± 0.01 e
3x − 2	20.79 ± 0.40 b	9.98 ± 0.22 e	2.10 ± 0.19 a	0.13 ± 0.004 cd	6.94 ± 0.21 b	0.56 ± 0.01 e
3x − 3	16.23 ± 0.32 g	7.62 ± 0.32 g	2.23 ± 0.49 a	0.14 ± 0.004 b	5.74 ± 0.21 cde	0.49 ± 0.01 g
Diploid (2x)	22.10 ± 0.62 a	11.73 ± 0.34 b	1.90 ± 0.21 b	0.07 ± 0.003 h	7.92 ± 0.39 a	0.51 ± 0.02 fg

Values within same columns (or within same lines) followed by the same letter were not significantly different according to Tukey's HSD test ($P < 0.05$)

Overall, sucrose resulted in significantly better regeneration than maltose (12.3% vs. 8.2%, respectively) (Table 4). Maltose was a poor carbon source for most clones, with the exception of 3x − 1. In general, sucrose at 30 g l^{-1} resulted in the best regeneration, with the exception of 3x − 1, where 25 g l^{-1} was the best treatment. For Mix − 1, 30 g l^{-1} of either maltose or sucrose gave similar regeneration. There was either no shoot regeneration or decreased shoot regeneration for other neopolyploids and the diploid on SIM with maltose or reduced sucrose concentration. The neopolyploid clone 4x − 4 failed to regenerate in any treatment. These results showed that shoot organogenesis of different polyploid clones in response to carbon source and its concentration was highly genotype-dependent.

Ploidy level and phenotypic variation of regenerants derived from neopolyploids

Regenerated shoots from leaf explants of neopolyploids showed variation of phenotype and ploidy level, depending on the ploidy of the donor plant. Regenerants of triploid and tetraploid origin had morphological characteristics similar to the donor plants and had the same ploidy level as donor plants (Table 2). This indicated that the organization and function of the genome of triploids and tetraploids in this study were stably propagated through somatic cell divisions, suggesting mitotic fidelity in these neopolyploids. Regenerants from the pentaploid 5x − 2 and the mixploid Mix − 1 showed variation in both phenotype and ploidy level. Regenerants from pentaploids 5x − 1 and 5x − 2 were uniform. Sixty-three regenerants from 5x − 1 all had the same phenotype and ploidy level as the source pentaploid. Forty-nine regenerants from 5x − 2 all had phenotypes and ploidy levels different from the parent pentaploid, but all had the same phenotype and ploidy level as the original diploid. This suggests that the genome of the diploid progenitor of this pentaploid clone was stable (maybe only chromosome number multiplication), and that no chromosomal variance or gene mutation in somatic cells of this pentaploid clone occurred. The diploidization of derivatives of this pentaploid clone indicated that this neopolyploid is mitotically unstable. This result is in agreement with the findings of mitotic instability in allopolyploids of *Arabidopsis* (Wright et al. 2009) and *Rubus* (Britton and Hull 1957). The regenerants from Mix − 1 (4x/6x) were separated into two types: triploid (Fig. 2b) and hexaploid, suggesting that the presence of multiple homologous chromosomes resulted in spurious pairing between chromosomes and unpaired chromosomes and daughter cells with unbalanced chromosome numbers (aneuploids). Aneuploid cells grow more slowly and are overtaken by the preferential proliferation of surrounding euploid cells (Comai 2005).

The phenotype of triploid clone 3x − 3 (Fig. 1j and Fig. 2b) derived from mixploid Mix − 1(Fig. 1i and Fig. 2f) was very different from Mix − 1 and the other neopolyploids with same progenitor, suggesting that polyploidization maybe accompany chromosomal structure variation or gene mutation, resulting in transgressive (non-additive) gene regulation (Comai 2005).

Discussion

Polyploids generally differ markedly from their progenitors in morphological, ecological, physiological and cytological characteristics that can contribute both to exploitation of a new niche and to reproductive isolation (Ramsey and

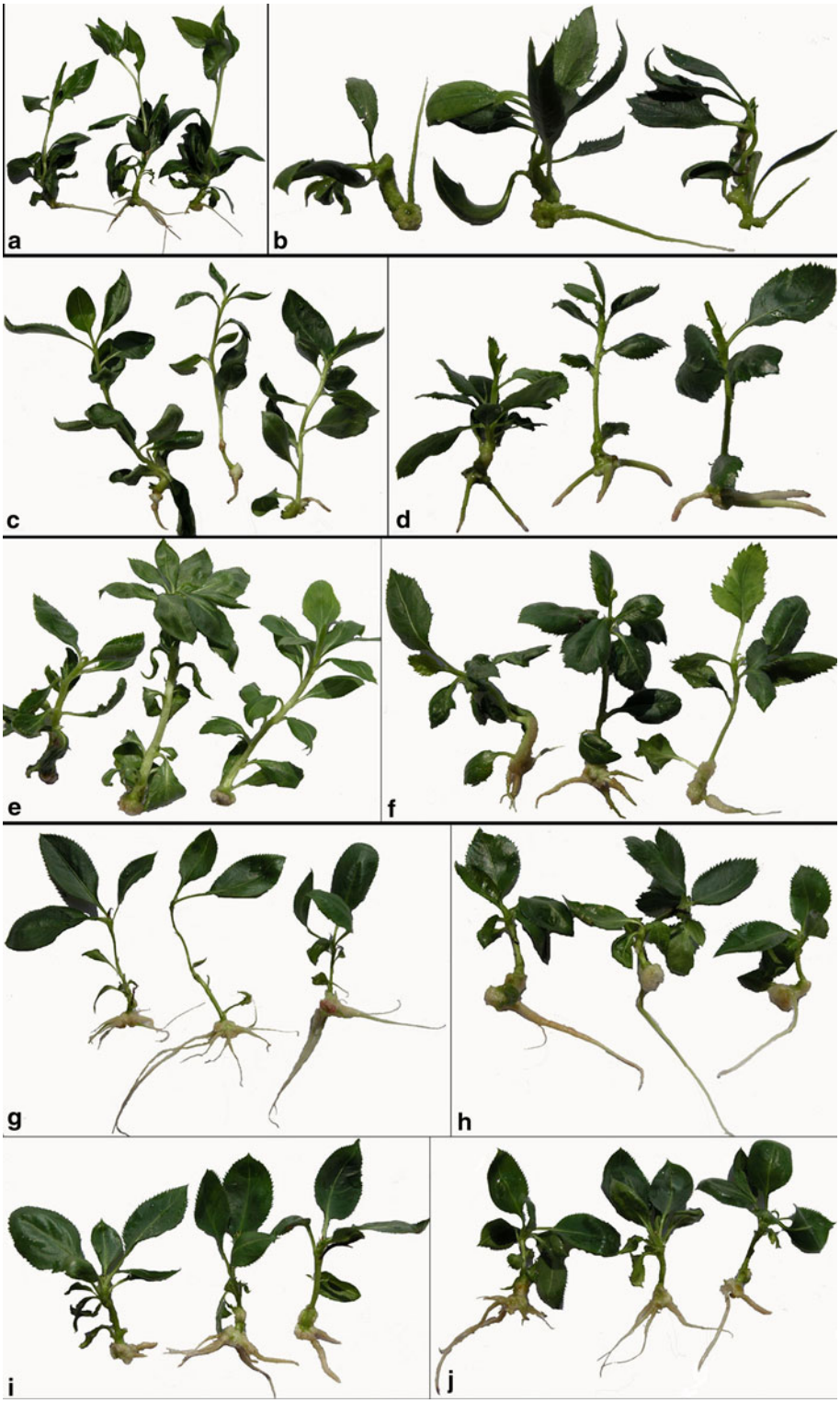

Fig. 2 Shoot growth of different ploidy clones of pear on rooting medium (1/2-strength QL medium supplemented with 0.5 mg l^{-1} IBA, 1 g l^{-1} polyvinyl alcohol (PVA) and 20 g l^{-1} sucrose). **a** and **b** triploid clones 3x − 2 and 3x − 3 **c**: tetraploid clone 4x − 4 **d** and **e** pentaploid clones 5x − 1 and 5x − 2 **f** mixploid clone Mix − 1 **g** diploid 'Fertility' **h** triploid clone 3x − 1 **i** and **j** tetraploid clones 4x − 1 and 4x − 2

Schemske 2002). In this paper, a wide range of phenotypic variation of neopolyploids was observed. Phenotypic differentiation may be driven by polyploidy per se, through the combined effects of increased cell size, gene dosage effects, allelic diversity (Levin 1983), altered regulatory networks (Osborn et al. 2003), more complicated rearrangements of chromosomes or small changes in the structure of chromosome, such as deletion or duplication of a copy (copies) of a gene, which altered expression of specific genes. Compared to its donor diploid clone,

Table 2 Effect of different ploidy clones on shoot regeneration from leaf explants and growth characteristics comparison between regenerated shoots and clone neopolyploids

Ploidy clone	Shoot regeneration rate (%)*	Regenerated shoots growth characteristics in vitro	Regenerated shoots ploidy level
Mix − 1	6.7 ± 2.9 c	Separated into two types: one type is markedly different from donor clone; another type is similar to donor clone	Triploid and hexaploid
5x − 1	11.7 ± 7.6 b	Uniform, similar to donor clone	Pentploid
5x − 2	16.1 ± 12.1 bc	Not separated, but markedly different from donor clone and similar to original dipoid	Diploid
4x − 1	61.7 ± 14.4 a	Uniform, similar to donor clone	Tetraploid
4x − 2	32.9 ± 6.7 b	Uniform, similar to donor clone	Tetraploid
4x − 3	63.2 ± 12.8 a	Not separated, similar to donor clone	Tetraploid
4x − 4	0 ± 0 c	Failed to regenerate	N/A
3x − 1	16.7 ± 5.8 bc	Uniform, similar to donor clone	Triploid
3x − 2	6.7 ± 2.9 c	Uniform, similar to donor clone	Triploid
Diploid control	83.3 ± 5.8 a	Uniform, similar to donor clone	Diploid

* Shoot induction medium was NN69 with 5 mg l^{-1} BA and 0.5 mg l^{-1} IBA

Means followed by different letters within same column were significantly different at $P < 0.05$ by Tukey's HSD test

Table 3 Effect of media on percentage shoot regeneration from leaf explants of different neopolyploids

Neopolyploid Clone	Shoot induction media*		
	NN69 + 1 mg·l^{-1} TDZ	NN69 + 2 mg·l^{-1} TDZ	1/2 MS + 5 mg·l^{-1} BA
Mix − 1	3.3 ± 3.3 a	3.3 ± 3.3 a	0.0 ± 0.0 a
5x − 1	13.3 ± 3.3 a	1.7 ± 1.7 b	23.3 ± 6.7 a
5x − 2	0.0 ± 0.0 a	2.8 ± 1.5 a	0.0 ± 0.0 a
4x − 2	38.3 ± 4.4 a	35.0 ± 2.9 ab	30.0 ± 11.5 b
4x − 4	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
3x − 1	15.0 ± 5.0 b	31.7 ± 7.3 a	16.7 ± 8.8 b
3x − 2	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
Medium Mean	10.0 ± 3.1 a	10.6 ± 3.4 a	10.0 ± 3.3 a

* All media contain 0.5 mg l^{-1}IBA

Means for each clone followed by different letters in a row were significantly different at $P < 0.05$ by Tukey's HSD test

Means for each medium followed by different letters in the last row were significantly different at $P < 0.05$ by Tukey's HSD test

Table 4 Effect of carbon source on shoot regeneration from leaf explants of neopolyploids and diploid control

Carbon source (g l^{-1})	Neopolyploid clones and diploid control (means ± SE)							
	Mix − 1	5x − 1	5x − 2	4x − 2	4x − 4	3x − 1	3x − 2	Diploid 2x
Maltose 20	0.0 ± 0.0 a	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 a	23.1 ± 4.2 b	0.0 ± 0.0 a	20.0 ± 10.0 c
Maltose 25	3.3 ± 2.9 a	0.0 ± 0.0 b	0.0 ± 0.0 b	6.7 ± 5.8 b	0.0 ± 0.0 a	46.9 ± 5.0 a	0.0 ± 0.0 a	30.0 ± 10.0 b
Maltose 30	5.0 ± 5.0 a	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 a	48.9 ± 1.9 a	0.0 ± 0.0 a	13.3 ± 5.8 c
Sucrose 20	0.0 ± 0.0 a	0.0 ± 0.0 b	0.0 ± 0.0 b	3.3 ± 2.9 b	0.0 ± 0.0 a	21.7 ± 2.9 b	0.0 ± 0.0 a	20.0 ± 10.0 c
Sucrose 25	0.0 ± 0.0 a	0.0 ± 0.0 b	0.0 ± 0.0 b	6.7 ± 2.9 b	0.0 ± 0.0 a	45.0 ± 13.2 a	0.0 ± 0.0 a	25.0 ± 5.0 bc
Sucrose 30	6.7 ± 2.9 a	11.7 ± 7.6 a	16.1 ± 12.1 a	32.9 ± 6.7 a	0.0 ± 0.0 a	16.7 ± 5.8 b	6.7 ± 2.9 a	83.3 ± 5.8 a
Clone least square means ± SE	2.5 ± 1.2 c	1.9 ± 1.2 c	2.7 ± 1.2 c	8.3 ± 1.2 b	0.0 ± 1.2 c	33.7 ± 1.2 a	1.1 ± 1.2 c	31.9 ± 1.2 a

Clone by carbon source by concentration means within same columns followed by the same letter were not significantly different according to Tukey's HSD test ($P < 0.05$). Clone means followed by the same letter in the last row were not significantly different according to Tukey's HSD test ($P < 0.05$)

phenotypic variation in different neopolyploid clones in this study were probably caused by changes in ploidy levels, as reported in potato (Ramulu et al. 1983; Li et al. 1992), poplar and black locust (Ewald et al. 2009) and in *Phragmites australis* (Paucã-Comãnescu et al. 1999), but the gene dosage effects in these cited studies were not evident in our study, with the possible exception of ploidy means for petiole width. Phenotypic variation among different clones with the same ploidy level were observed in this study, for example, triploids 3x − 1 (Fig. 1d) and 3x − 3 (Fig. 1j), tetraploids 4x − 1 (Fig. 1b) and 4x − 4 (Fig. 1f), pentaploids 5x − 1 (Fig. 1g) and 5x − 2 (Fig. 1h), indicating that doubling of chromosomes not only resulted in chromosome and gene redundancy, but perhaps that chromosomal rearrangements, inversions, and translocations occurred following genome duplication, or that loss or retention of the duplicated genes occurred, as previously reported for recent polyploidy of *Arabidopsis thaliana* (compared to ancient polyploidy) (Blanc and Wolfe 2004). In that case, duplicated genes were not all equal regarding their loss or retention in the genome, and several functional categories were found for which duplicated genes have been preferentially kept or lost. The duplication of a regulatory gene is likely to influence phenotypic traits. Ploidy levels of neopolyploids in this paper were estimated by flow cytometry, which was effective in differentiating chromosome numbers differing by 1x, but was not able to differentiate aneuploids. So, phenotypic differentiation of different clones due to aneuploidy cannot be excluded. The chromosome numbers of polyploids need to be further determined. The molecular mechanism of phenotypic variation of different neopolyploid genotypes in this paper was not known.

In this study, in vitro shoot and root organogenesis of most of the induced neopolyploids were significantly decreased compared to the original diploid. This result is in conformity with the previous report that polyploidization of cucumber callus cultures was accompanied by a gradual loss of regeneration ability. Plants regenerated from callus cultures were classified as diploid (57%), tetraploid (18%), octoploid (4%) and mixoploid (2n/4n, 4%; 4n/8n, 17%) (Kubalakova et al. 1996). The decrease of in vitro organogenic ability of polyploids is possibly due to the difficulty of normal completion of mitosis or the propensity to produce aneuploid cells during mitosis. Aneuploid cells grow slowly and are overgrown by the preferential proliferation of surrounding euploid cells (Comai 2005). The difference of shoot regeneration capacity and ploidy variation of regenerants from somatic cells of neopolyploids confirmed that the complexity of managing and partitioning chromosomes during cell mitotic division.

The absence of shoot organogenesis from leaf explants of the neopolyploid clone 4x − 4 showed the loss of in vitro totipotency. The results support the previous finding that the loss of totipotency in vitro was often related to polyploidization (Colijn-Hooymans et al. 1994; Kubalakova et al. 1996). The *tan* mutant in *Arabidopsis* could not initiate shoot organogenesis from somatic tissue on CIM-SIM media (Baster et al. 2009), suggesting the involvement of the *TAN* gene in basic cellular processes related to cell growth and differentiation. There is little information on the link between the loss of in vitro organogenesis and polyploidization in plant. But in yeast, the sensitivity of mitotic chromosomal segregation to ploidy was found to be due to ploidy-conditional lethality in the gene that encodes the microtuble-associated protein BIKI. BIKI is required for normal cytoskeletal function and its loss has no major consequences in diploids but results in mitotic lethality in tetraploids (Lin et al. 2001). Another interpretation should be considered. The maximum ploidy level that a species could tolerate seems limited, but the mechanism(s) that determine the limit is unknown.

The differences in shoot regeneration on the same SIM and the different responses to carbon source of neopolyploids showed the diversity of responses among the neopolyploids. Instead of freezing a species in a static state, polyploids have the ability to diversify gene function by altering redundant copies of important or essential genes (Comai 2005). The difference of organogenic capacity of different ploidy levels and different genotypes of the same ploidy level derived from the same diploid donor plants showed the functional divergence of duplicated genes formed by neopolyploids.

On the same basal medium (NN69), plant growth regulator TDZ was less effective for adventitious shoot regeneration of neopolyploids than BA, exclusive of 3x − 1 (Table 2 and 3), similar to the result in wild pear (Caboni et al. 1999). In this study, polyploid clones 3x − 2, 4x − 2 and 4x − 4 showed no significant differences in shoot regeneration percentage between basal medium NN69 and 1/2 MS when supplemented with same cytokinin (BA), similar to a previous report that there was no significant difference in shoot regeneration percentage between NN69 and 1/2 MS salts for pear cultivars 'Seckel' and 'Louise Bonne Panachee' when the same two media were compared (Abu-Qaoud et al. 1991). But other polyploid clones Mix − 1, 5x − 1, 5x − 2 and 3x − 2 showed different shoot regeneration percentages. The results showed that regeneration potential was influenced by a genotypic effect.

In conclusion, neopolyploids obtained by in vitro induced leaf explants of pear diploid cultivar 'Fertility' showed a wide range of phenotypic variation, especially in leaf traits, such as leaf index, leaf colour and leaf thicknees. Compared to its diploid, generally, neopolyploids have either larger values of leaf index or smaller values of leaf index or more darker leaf colour or more thicker leaf

thickness or have thicker leaves with darker colour, similar to the report that polyploids showed visible differences such as thicker leaves and modified leaf morphology (Ewald et al. 2009). The organogenic ability of most neo-polyploids decreased compared to its donor diploid, similar to previous research in somatic embryogenesis of callus culture that concluded that polyploidization of cells had a strict relationship with decrease and loss of totipotency in vitro (Kubalakova et al. 1996). However, in this paper, the molecular mechanism of decrease or loss of in vitro organogenesis of cultured somatic tissue of pear neopolyploid is not clear and needs to be elucidated.

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